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L2	L1 and (bismuth adj 213)	37	L2
L1	(treat$ or inhibit$)with (cancer or tumor or neoplas$)	54719	L1

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☐ 1. Document ID: US 5612016 A

L4: Entry 1 of 4 File: USPT Mar 18, 1997

US-PAT-NO: 5612016
DOCUMENT-IDENTIFIER: US 5612016 A

TITLE: Conjugates of antibodies and bifunctional ligands

DATE-ISSUED: March 18, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Griffiths; Gary L.	Morristown	NJ		
Diril; Habibe	Paterson	NJ		
Hansen; Hans J.	Mystic Island	NJ		

US-CL-CURRENT: 424/1.49; 424/1.53, 530/391.3, 530/391.5, 530/402, 530/408, 530/409

ABSTRACT:

Provided are conjugates useful in cancer, cardiovascular or infectious disease detection and/or therapy. The conjugate is of a ligand and protein. The ligand has a moiety capable of binding to mercapto groups and is capable of chelating a metal useful for detection or therapy. The protein reacts with a substance associated with a targeted cell, pathologic lesion or pathogen. The protein prior to conjugation has at least one mercapto group which becomes a site for conjugation to the ligand. Also provided are metal chelates of the conjugate, methods of detection and therapy, methods for producing the conjugate and pharmaceuticals compositions of the conjugates.

16 Claims, 0 Drawing figures
Exemplary Claim Number: 1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

☐ 2. Document ID: US 5482698 A

L4: Entry 2 of 4 File: USPT Jan 9, 1996

US-PAT-NO: 5482698
DOCUMENT-IDENTIFIER: US 5482698 A

TITLE: Detection and therapy of lesions with biotin/avidin polymer conjugates

DATE-ISSUED: January 9, 1996

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Griffiths; Gary L.	Morristown	NJ		

US-CL-CURRENT: 424/1.41; 424/1.45, 424/1.49, 424/1.69, 424/78.08, 424/9.34, 424/9.35, 424/9.36, 424/9.4, 424/9.6, 514/387

ABSTRACT:

Methods of detecting and/or treating lesions in a patient are provided. The methods are an improvement over known methods comprising the steps of (a) parenterally injecting a subject with a targeting composition comprised of a biotin-protein conjugate or an avidin-protein conjugate, wherein the protein preferentially binds to a marker substance produced or associated with the targeted lesion, and allowing the protein conjugate to preferentially accrete at the targeted lesion; (b) then parenterally injecting a clearing composition comprised of (i) avidin, when the targeting composition is a biotin-protein conjugate, or (ii) biotin, when the targeting composition is a avidin-protein conjugate, and allowing the clearing composition to substantially clear the targeting composition from non-targeted sites and to bind to the targeting composition accreted at the targeted lesion; and (c) parenterally injecting a detection or therapeutic composition comprised of a conjugate of (i) avidin and detection or therapeutic agent when the clearing composition is biotin, or (ii) biotin and detection or therapeutic agent when the clearing agent is avidin, and allowing the composition to accrete at the targeted lesion. The improvement is having at least one of the compositions of step (a) or (b) further comprise a polymer to which multiple moieties of avidin or biotin can conjugate, thereby providing an increased number of binding sites to which a subsequently administrated composition can bind thereby amplifying the amount of detection or therapeutic agent at the targeted site.

43 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 3. Document ID: US 5047227 A

L4: Entry 3 of 4 File: USPT Sep 10, 1991

US-PAT-NO: 5047227
DOCUMENT-IDENTIFIER: US 5047227 A

TITLE: Novel and improved antibodies for site specific attachment of compounds

DATE-ISSUED: September 10, 1991

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rodwell; John D.	Yardley	PA		

McKearn; Thomas J.	New Hope	PA
Long; Cynthia G.	Skillman	NJ

US-CL-CURRENT: 424/1.49; 424/1.53, 424/178.1, 424/181.1, 424/9.34, 530/322, 530/391.5, 530/391.9, 530/395

ABSTRACT:

Novel and improved antibody compositions comprising an antibody or antibody fragment having an increased number of sites on a carbohydrate side chain available for the site specific attachment of a compound to a region of the antibody or antibody fragment which is not part of nor directly involved with the antigen binding site are disclosed. Conjugates, prepared using the high-mannose-containing antibodies, which are characterized by substantially the same immunospecificity as the unmodified unconjugated antibody molecule, are also disclosed. The antibody compositions are advantageously used for a wide variety of therapeutic and diagnostic applications in both in vivo and in vitro targeted delivery systems and assays. Methods for preparing the antibody compositions, as well as antibody conjugate intermediates, and methods for using the antibody compositions are also described.

16 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 4. Document ID: US 4454106 A

L4: Entry 4 of 4 File: USPT Jun 12, 1984

US-PAT-NO: 4454106
DOCUMENT-IDENTIFIER: US 4454106 A
**** See image for Certificate of Correction ****

TITLE: Use of metal chelate conjugated monoclonal antibodies

DATE-ISSUED: June 12, 1984

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gansow; Otto A.	Washington	DC	20008	
Strand; Mette	Baltimore	MD	21210	

US-CL-CURRENT: 424/1.53; 424/9.34, 436/548, 436/73, 436/74, 436/804, 436/819, 436/84, 600/3, 600/436

ABSTRACT:

Therapeutic and diagnostic methods employing metal chelate conjugated monoclonal antibodies are described. Metals employed in therapeutic conjugated antibodies include alpha particle, beta particle or Auger electron emitting isotopes. Diagnostic methods may be either in vivo or in vitro. Chelated metals employed in diagnostic techniques may include, inter alia, gamma or positron emitting metals as

well as fluorogenic or paramagnetic metals.

21 Claims, 0 Drawing figures
Exemplary Claim Number: 15,18

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw. De

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L3	L2 and antibod$	35	L3
L2	L1 and (bismuth adj 213)	37	L2
L1	(treat$ or inhibit$)with (cancer or tumor or neoplas$)	54719	L1

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15839464   BIOSIS NO.: 200100011303
An alpha-particle emitting antibody ((213Bi)J591) for radioimmunotherapy of
  prostate cancer
AUTHOR: McDevitt Michael R; Barendswaard Els; Ma Dangshe; Lai Lawrence;
  Curcio Michael J; Sgouros George; Ballangrud Ase M; Yang Wei-Hong; Finn
  Ronald D; Pellegrini Virginia; Geerlings Maurits W Jr; Lee Mona;
  Brechbiel Martin W; Bander Neil H; Cordon-Cardo Carlos; Scheinberg David
  A (Reprint)
AUTHOR ADDRESS: Memorial Sloan-Kettering Cancer Center, 1275 York Avenue,
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JOURNAL: Cancer Research  60 (21): p6095-6100 November 1, 2000 2000
MEDIUM: print
ISSN: 0008-5472
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: A novel alpha-particle emitting monoclonal antibody construct
  targeting the external domain of prostate-specific membrane antigen
  (PSMA) was prepared and evaluated in vitro and in vivo. The chelating
  agent,
  N-(2-amino-3-(p-isothiocyanatophen-yl)propyl)-trans-cyclohexane-1,2-diami
  ne-N,N',N',N'',N''-pentaacetic acid, was appended to J591 monoclonal
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antibody to stably bind the 213Bi radiometal ion. Bismuth-213 is a short-lived (t1/2 = 46 min) radionuclide that emits high energy alpha-particles with an effective range of 0.07-0.10 mm that are ideally

European Journal of Surgical Oncology 1996; **22**: 331–334

Technetium-99m tetrofosmin scintigraphy in the diagnosis of breast cancer

M. R. Vieira* and J. H. B. Weinholtz†

**Department of Nuclear Medicine, and* †*Department of Surgery, Instituto Português de Oncologia, Lisboa, Portugal*

The routine mammogram remains the procedure of choice for screening asymptomatic women for breast cancer. However, it accounts for an elevated percentage of misdiagnosed breast nodules particularly in younger women with dense breast tissue or fibrocystic disease. New radiopharmaceuticals including labelled monoclonal antibodies, thalium-201 and technetium-99m sestamibi are under investigation as possible solutions to this issue. 99mTc-tetrofosmin (Myoview), like 99mTc-sestamibi, is a lipophilic, cationic myocardial imaging agent. Mitochondrial membrane potential appears to play a significant role in the uptake and/or retention of 99mTc-tetrofosmin. Twenty-seven female patients with suspected breast cancer were included in the study. Twenty mCi of 99mTc-tetrofosmin were administered intravenously. Imaging was begun 10 min post-injection. In twenty-three patients, breast cancer was detected using 99mTc-tetrofosmin scintigraphy. Two carcinomas were missed. In two patients, both breast scintigraphy and intraoperative biopsies were negative. Our preliminary results suggest that this product may have a role in the differentiation of benign from malignant lesions, and were encouraging in terms of its potential usefulness as a marker of malignancy.

Key words: **technetium-99m tetrofosmin; breast cancer; tumour viability.**

Introduction

Breast cancer is a major health problem in developed countries. The primary purpose of screening asymptomatic women for breast cancer is to diagnose breast cancer in an early stage and to reduce mortality.[1] At present early detection relies on patient self-examination, physical examination in a medical setting and mammography. In addition, mammography is currently the only reliable method to detect non-palpable breast cancer.[2] However, it has been reported that the mammographic technique is less reliable for detecting lesions in the dense or dysplastic breast.[3] Mammography also may be of limited value when only heterogeneity or microcalcifications without mass are present.[4] Currently, the only established method to resolve such a dilemma is random tissue sampling which is usually attended by high non-malignant to malignant biopsy rates. Breast biopsy entails considerable physical and psychological side effects. Furthermore, the economic cost of these interventions is high.

Clearly, there is a great need for the development of new and reliable methods for diagnosis of breast cancer to complement the existing diagnostic procedures of palpation, mammography and ultrasonography. A non-invasive method to select patients who would benefit most from breast biopsy and reduce the number of negative biopsies would clearly be of value.

New radiopharmaceuticals including thallium-201 and 99mTc-sestamibi are under investigation to address this issue.[5–7]

99mTc-tetrofosmin is a labelled diphosphine compound and like technetium-90m methoxyisobutylisonitrile (99mTc-MIBI) is a lipophilic cation suitable for myocardial imaging. Experimental results suggest that the cellular uptake of technetium-tetrofosmin is driven by the sarcolemmal and mitochondrial transmembrane potentials. Even if blood flow is a limiting factor, tissue retention can occur if these potentials are preserved (viable cells).[8]

The main purpose of the current prospective study is to assess the sensitivity of 99mTc-tetrofosmin breast scintigraphy in tumour detection and nodal involvement in a group of women with mammograms suspicious for breast malignancy.

Materials and methods

Patients

Twenty-seven female patients (age range: 27–80 years) with suspect mammograms underwent 99mTc-tetrofosmin breast scintigraphy in order to assess the value of 99mTc-tetrofosmin scans in the detection of breast carcinoma. Scans were classified as positive (abnormal focal accumulation at the tumour site) or negative (no abnormal focus of activity

Correspondence to: Maria do Rosário Vieira, Serviço de Medicina Nuclear, Instituto Português de Oncologia, Rua Prof. Lima Basto, 1093 Lisboa Codex, Portugal.

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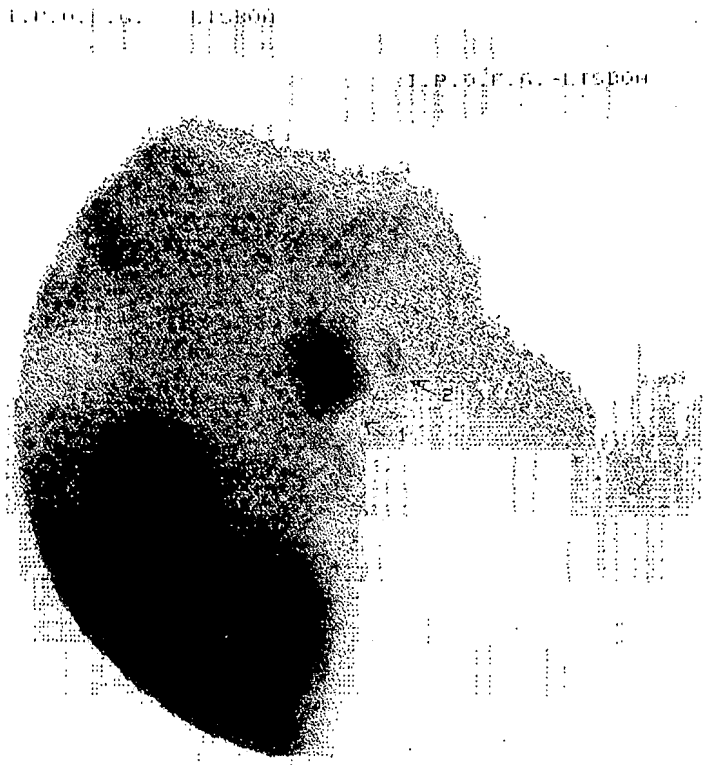

Fig. 1. Patient 6: a 43-year-old female with an infiltrating ductal cell carcinoma of the left breast 1.2 cm on the pathological section and a palpable lymph node in the left axilla measuring 0.5 cm.

at the tumour site). The data were considered in two groups of patients—Group A: 21 patients with palpable breast masses; Group B: Six patients with mammography demonstrating abnormalities such as calcifications or suspicious masses in which the referring physician could not demonstrate a palpable nodule. Breast scintigraphy was performed before surgery, biopsy or aspiration cytology.

Informed consent was obtained from each patient.

99mTc-tetrofosmin scintigraphy

A single dose of 20 mCi of 99mTc-tetrofosmin was given intravenously in the antecubital vein of the arm contralateral to the tumour. The labelling and quality control procedures were carried out according to the manufacturer's instructions. Labelling efficiency was always higher than 90%. Scans were performed at 10 min post-injection.

Images were taken with a STARPORT-GE gamma camera, provided with a low-energy, high-resolution collimator. A first set of scans included the anterior, lateral and oblique views in supine imaging with the field of view angled to include the axilla. If necessary we conducted prone lateral and oblique views. The arms were raised with the hands placed behind the patient's head when possible. Regions of interest (ROIs) of the same areas were drawn on the tumour and background in the same breast. We determined the ratio between average tumour counts and average background counts. All scintigraphic results were interpreted without previous knowledge of the histological data.

Results

In 21 patients with palpable breast carcinoma (Group A) 20 adenocarcinomas (95%) were detected using 99mTc-tetrofosmin scintigraphy. One small carcinoma, 8 mm in diameter, was missed. This lesion was located in the right breast, and had no positive axillary nodes after surgery.

Among the six patients (Group B) with suspicious mammograms (microcalcifications, ductal asymmetry, focal architectural distorsion), but without palpable masses, breast scintigraphy failed to reveal abnormalities in three. Two were indeed negative on excisional biopsy. The false-negative result was a ductal carcinoma in the right breast. The diagnostic sensitivity (TP/TP + FN) in this small group of patients was 75%. Two mammograms produced false-negative results. The sensitivity of mammography was 92.6%.

Eleven patients were demonstrated to have lymph node metastases at the time of surgery. In seven patients scintimammography demonstrated axillary nodes (Fig. 1)

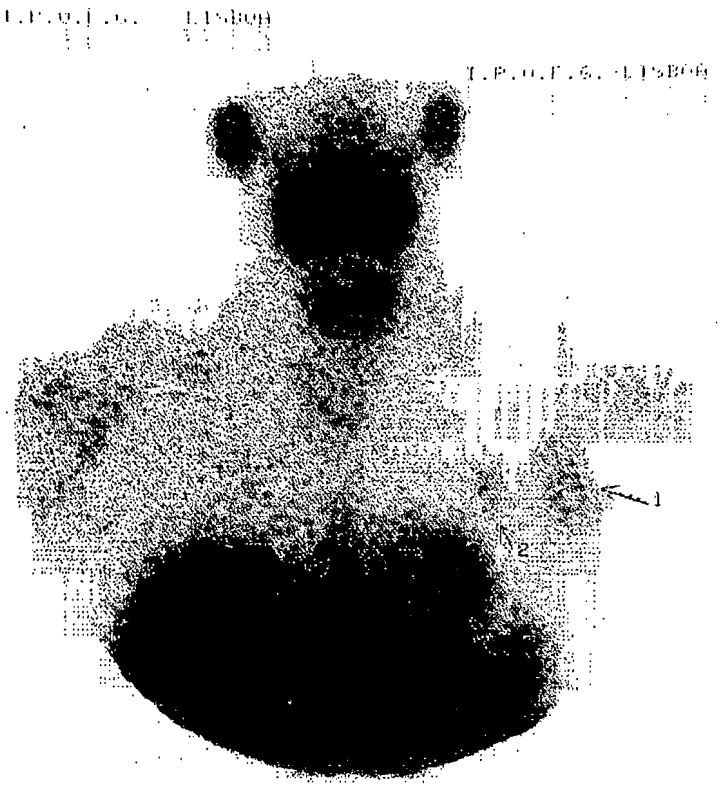

Fig. 2. Patient 5: a 49-year-old female who had a palpable nodule in her left breast suspicious for malignancy on mammography. Scintigraphy shows the primary tumour (1), and a lymph node (2), measuring 4.5 cm and 1.2 cm respectively on pathological section.

and in one patient positive nodes in the internal mammary chain were detected (Fig. 2).

In the remaining four patients, no palpable nodes were present and only after surgery was their presence histologically confirmed (Table 1).

We determined the lesion/background ratios in 14 exams, the minimum value being 1.14, with a mean of 1.32.

Discussion

The role of mammography in the detection of breast cancer in women with dense fibroglandular patterns is far from being accepted.[9] It frequently cannot be used to differentiate accurately benign from malignant lesions.[10] Therefore most biopsies that are performed as a result of abnormal mammograms are negative.[9] In this clinical field, nuclear medicine techniques could play an important role. In the last decade tumour imaging agents have been developed and some have achieved widespread acceptance in clinical imaging.[11]

The cellular uptake mechanism of 99mTc-tetrofosmin is probably related to regional blood flow and mitochondrial content as described for 99mTc-MIBI.[7]

Our study protocol was similar to that performed by Kao *et al.*[7] Twenty-seven women were found by mammography to have breast abnormalities highly suspicious of malignancy and referred for biopsy or surgery. Breast imaging was done 10 min after the radiopharmaceutical administration. In women with large and dense breasts and with small deep breast lesions we conducted prone scintigraphy lateral and oblique views.[12]

All, except one, of the palpable breast lesions were detected in our study. The false-negative result was a tumour measuring 0.8 cm. However, one breast cancer of 0.4 cm was visualized by 99mTc-tetrofosmin scintigraphy (case 2). All palpable lymph nodes were also positive in the scintimammography, but we could not image those small nodes that only diagnosed as positive in histology after being removed surgically. In a total of 11 patients with axillary metastases, eight of 15 nodules were identified (53%). The percentage of detection of lymph node metastases in the study conducted by Kao *et al.*[7] using 99mTc MIBI was 67% (eight of 12 nodules were demonstrated). It seems that this method would not be useful in evaluating the axilla for detection of lymph node metastases.

Recently Tarik published the results of 99mTc-tetrofosmin imaging in five patients with bronchial carcinoma.[13] The ratios between tumour/non-tumour were similar to those found in our study and the peak concentration at the tumour was reached at the end of the first minute post-injection.

Table 1. Patients

Case no.	Age (years)	Pathology report	Mammography	Tumour side	Tumour size (cm)	Axillary lymph node metastases	99mTc-tetrofosmin uptake	
							Tumour	Axillary lymph nodes
1	60	Benign	Suspicious	Right	—	Absent	Negative	Negative
2	66	Adenocarcinoma	Suspicious	Right	0.4	Absent	Positive	Negative
3	44	Adenocarcinoma	Nodule	Right	0.8	Absent	Positive	Negative
4	48	Adenocarcinoma	Nodule	Right	1.4	Absent	Positive	Negative
5	49	Adenocarcinoma	Nodule	Left	4.5	Present	Positive	Positive
6	43	Adenocarcinoma	Nodule	Left	1.5	Present	Positive	Positive
7	44	Adenocarcinoma	Nodule	Right	1.5	Present	Positive	Negative
8	58	Adenocarcinoma	Nodule	Left	1.5	Absent	Positive	Negative
9	58	Adenocarcinoma	Nodule	Left	2.5	Present	Positive	Positive
10	80	Adenocarcinoma	Nodule	Left	0.9	Absent	Positive	Negative
11	66	Adenocarcinoma	Nodule	Left	1.7	Absent	Positive	Negative
12	64	Adenocarcinoma	Suspicious	Right	0.7	Absent	Negative	Negative
13	56	Adenocarcinoma	Nodule	Right	2.5	Absent	Positive	Negative
14	49	Adenocarcinoma	Nodule	Right	4.0	Present	Positive	Positive
15	60	Adenocarcinoma	Nodule	Left	3.0	Absent	Positive	Negative
16	67	Adenocarcinoma	Nodule	Left	2.1	Present	Positive	Positive
17	51	Adenocarcinoma	Nodule	Left	1.4	Absent	Positive	Negative
18	41	Adenocarcinoma	Nodule	Left	3.0	Present	Positive	Positive
19	59	Adenocarcinoma	Nodule	Left	2.4	Present	Positive	Negative
20	57	Adenocarcinoma	Nodule	Right	0.8	Absent	Negative	Negative
21	76	Adenocarcinoma	Suspicious	Left	1.0	Present	Positive	Negative
22	66	Adenocarcinoma	Nodule	Left	0.8	Absent	Positive	Negative
23	69	Adenocarcinoma	Nodule	Left	3	Present	Positive	Negative
24	71	Benign	Suspicious	Left	—	Absent	Negative	Negative
25	68	Adenocarcinoma	Nodule	Right	1.2	Absent	Positive	Negative
26	67	Adenocarcinoma	Suspicious	Right	1.6	Absent	Negative	Positive
27	27	Adenocarcinoma	Nodule	Left	2	Present	Positive	Positive

We conclude that scintimammography with 99mTc-tetrofosmin is a very simple, non-invasive, and rapidly performed technique. The current study demonstrates a high sensitivity (95%) for palpable breast malignancies and suggests a rationale in selecting patients for surgical biopsy. In patients with a high risk for the development of breast cancer, and who have mammograms considered difficult to interpret, a breast imaging study with 99mTc-tetrofosmin may help to determine which patients will benefit from excisional surgical biopsy.

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Accepted for publication 29 March 1996

Nuclear Medicine Communications (1995) 16, 468–476

Nuclear Medicine Communications

Cytotoxicity of ^{213}Bi- and ^{225}Ac-immunoconjugates

F.M. KASPERSEN,[1] E. BOS,[1]* A.V. DOORNMALEN,[1] M.W. GEERLINGS,[2] C. APOSTOLIDIS[3] and R. MOLINET[3]

[1]*NV Organon, PO Box 20, 5340 BH Oss,* [2]*Alphamedical Holding BV, Amsterdam, The Netherlands and* [3]*European Institute for Transuranium Elements, PO Box 2340, 7500 Karlsruhe, Germany*

Received 15 November 1994, in revised form 15 January 1995 and accepted 8 February 1995

Summary

This paper describes *in vitro* cytotoxicity experiments with ^{213}Bi- and ^{225}Ac-immunoconjugates on the human epidermoid tumour cell line A431 using a blood group A-reactive murine IgG (2D11) as the specific antibody and MOPC 21 as the control antibody. With both radionuclides, specific cell-killing was achieved. The observed cytotoxicity of ^{213}Bi ($T_{½}$ = 47 min) indicates that this radionuclide is a useful alternative for the α-emitter ^{212}Bi in the treatment of blood-borne malignancies. ^{225}Ac-immunoconjugates ($T_{½}$ of ^{225}Ac is 10 days) may be applicable for the treatment of solid tumours, since the daughter radionuclides of ^{225}Ac contribute to the cytotoxic efficacy by a field effect (i.e. toxicity in an area distal from the antibody-binding site). The lack of an adequate chelator for ^{225}Ac is a major drawback.

Introduction

Because of their extremely high cytotoxicity, short-lived α-emitting radionuclides are attractive candidates for application in radioimmunotherapy. In an earlier paper, we proposed ^{225}Ac as a source of α-particles for radioimmunotherapy. ^{225}Ac has a half-life of 10 days and decays into a cascade of short-lived α- and β-emitting radionuclides [1]. The last α-emitter of this series, ^{213}Bi, has a half-life of 47 min. This radionuclide might be a useful alternative for ^{212}Bi, which has been proposed for the treatment of blood-borne cancers [2, 3]. ^{225}Ac may be employed in radiotherapy with immunoconjugates of monoclonal antibodies that show a long residence time at the tumour site, such as the human IgM 16.88 [4], either using a one-step approach or via pretargeting schemes.

The radionuclides are readily available through milking from their precursor ^{229}Th ($T_{½}$=7340 years) and this radionuclide can be isolated, for example, from ^{233}U, prepared 30 years ago as fissile material for nuclear power reactors. The preparation of the ^{213}Bi and ^{225}Ac cows has been described elsewhere [1, 5]. In the present paper, the *in vitro* cytotoxicity of both radionuclides contained in immunoconjugates on human tumour cells will be presented.

*Author to whom all correspondence should be addressed.

Methods

SYNTHESIS OF BENZYL-DTPA

t-Butyl-(p-nitrobenzyl-diethylenetriamine-pentaacetate) 3

p-Nitrobenzyl-diethylenetriamine 2 was reacted with excess (15 eq.) *t*-butyl bromoacetate in refluxing ethanol in the presence of triethylamine (15 eq.) for 20 h. After removal of the salts by extraction, the product was purified by chromatography over Al_2O_3 (*n*-hexane/ethyl acetate, 85/15 v/v), yielding pure compound 3 as an oil in 45% yield. FAB-MS: m/z=809 ($[M+H]^+$). (The numbers 1–5 represent the final product and intermediates of the chemical analysis in Fig. 1.)

t-Butyl(-p-aminobenzyl-diethylenetriamine-pentaacetate) 4

This compound was prepared by reduction of 3 for 4 h in ethanol using H_2 (3 atm. pressure) with Pd/C (10%) as catalyst. After removal of the catalyst, the product was purified by chromatography over Al_2O_3 (*n*-hexane/ethyl acetate, 8/2 v/v), yielding pure 4 as an oil in 45% yield.

t-Butyl/p-isothiocyanotobenzyl-diethylenetriamine-pentaacetate) 5

Compound 5 was prepared by reaction of the amine 4 in dichloromethane with thiophosgene for 4 h at room temperature. The product was isolated as an oil in 50%

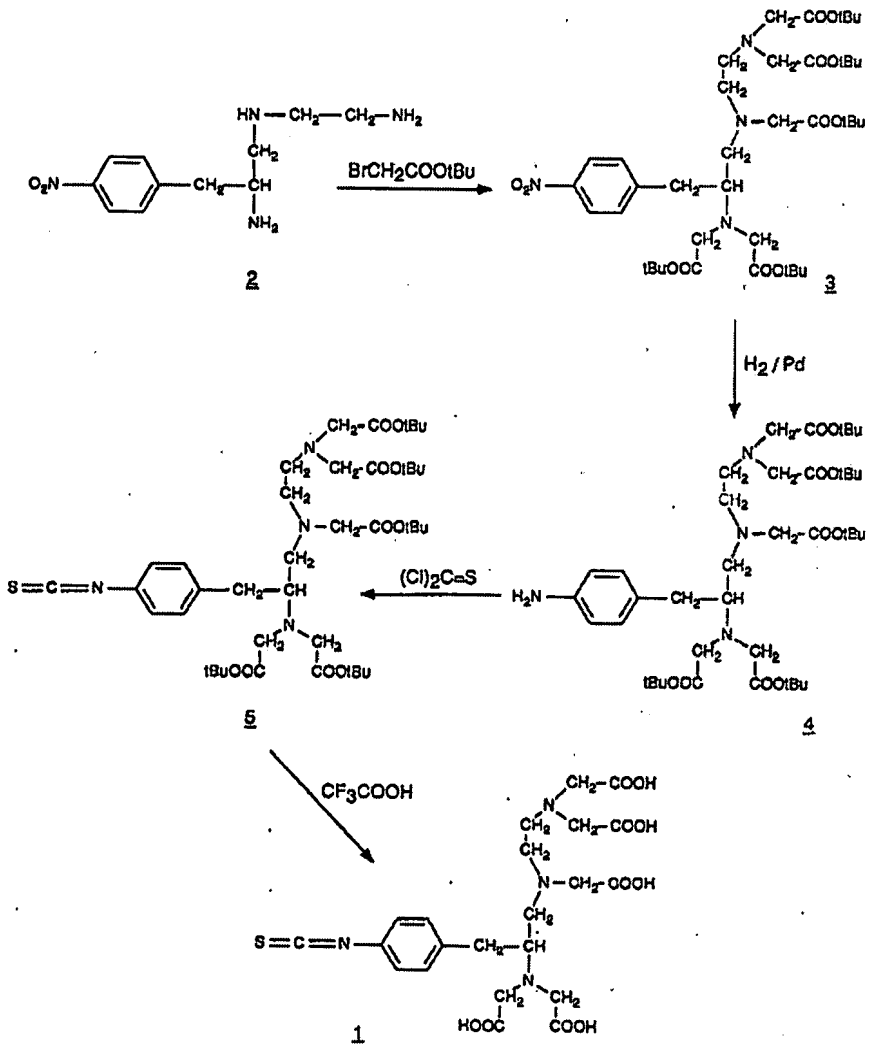

Fig. 1. Synthesis of benzyl-DTPA (compound 1). The numbers 1–5 represent the final product and intermediate compounds in the chemical synthesis.

yield by chromatography over Al_2O_3 (*n*-hexane/ethyl acetate, 85/15 v/v. Immunoreactivity (liquid): 2090 cm^{-1} (N=C=S); 1744 cm^{-1} (COOtBu). ^{1}H-NMR ($CDCl_3$): 1.40 ppm (tBu); 7.08 and 7.25 ppm (phenyl). FAB-MS: m/z= 821 ($[M+H]^+$) and m/z = 843 ($[M+Na]^+$).

p-Isothiocyanatobenzyl-diethylenetriamine-pentaacetate 1

The free chelating agent was prepared by reaction of 5 in trifluoroacetic acid (1 mg ml^{-1}) for 4 h at room temperature. The trifluoroacetic acid was removed by a gentle stream of nitrogen. The residue was dissolved in dichloromethane (1 ml) and again evaporated to dryness; this procedure was repeated three times leaving 1 as a crystalline white powder. FAB-MS: m/z = 539 ($[M-H]^-$) and m/z = 577 ($[M+K-2H]^-$).

PRODUCTION OF ^{225}Ac AND ^{213}Bi

Measurement of radioactivity

All radionuclides were measured by γ-spectroscopy. ^{225}Ac was measured on its 99.6 keV line or when in equilibrium with ^{213}Bi on the 440 keV line of ^{213}Bi. ^{221}Fr was measured on the 217.6 keV line and ^{225}Ra on its 40 keV line.

Production of ^{225}Ac and ^{213}Bi

In addition to the method described earlier [1], ^{225}Ac was also isolated from ^{225}Ra (obtained as a side-product from the ^{229}Th cow) in the following way. ^{225}Ra-fraction was stored for 15–20 days, after which time the amount of ^{225}Ac was at its maximum. After evaporation of the HNO_3, the Ra and Ac solution was dissolved in HCl (2 M, 200 µl) and fixed on a Dowex-column (50 W × 8; 100–200 mesh; 50 × 3 mm). ^{213}Bi and ^{209}Pb were washed

down with HCl (2 M, 3 column volumes) and ^{225}Ac was collected by elution with HNO_3 (4 M, 10 column volumes).

RECOIL EFFECTS OF ^{225}Ac-DECAY

Sepharose beads coated with human chorionic gonadotropin (hCG) were incubated in phosphate-buffered saline (PBS) for 60 min at ambient temperature with ahCG antibody chelated with ^{225}Ac. The beads were washed several times with buffer (isolation of beads by centrifugation) and incubated with buffer. After 5.15 and 30 min, samples from the buffer were taken and counted directly. As a control, samples of beads and supernatant were analysed in the same way.
same way.

SYNTHESIS OF IMMUNOCONJUGATES

Conjugation

The monoclonal antibodies (MAbs) to be conjugated – that is, the murine IgG1 antibodies SC-20 (aCEA), 2D11 (anti-blood group A), 130A (ahCG) and MOPC-21 (irrelevant control) – were transferred into PBS by chromatography on PD10. Prior to use, the PBS used in this procedure was chromatographed on Chelex in order to remove unwanted metal ion contaminants. Benzyl-DTPA (compound 4) was deprotected by incubation for 4 h in trifluoroacetic acid (TFA) at ambient temperature, then washed twice with CH_2Cl_2 and, finally, dissolved in PBS to a final concentration of approximately 5 mg ml^{-1}. Deprotected benzyl-DTPA was added to the antibody solutions in PBS at a molar antibody/chelator ratio of 1:10. The pH of the reaction mixture was adjusted to 8.9 with 0.1 M triethylamine. Incubation was performed for 2 h at 37°C. Excess reagent was removed by gel filtration on PD10 equilibrated in Chelex-treated PBS.

Determination of chelator content of DTPA conjugates

The determination of the DTPA content was carried out according to Pippin *et al.* [6]. To 500 µl of yttrium-arsenazo solution, 10 µl of conjugate solution in PBS was added and, after mixing, the absorbance at 652 nm was measured in a Pye-Unicam 8700 spectrophotometer. A calibration curve was prepared using 10 µl of DTPA solution in a concentration range of 20–100 nM.

A qualitative and quantitative analysis of chelator content of antibodies was carried out by ultrathin-layer isoelectric focusing (UTLIEF) at pH 3–9 in the Phast Electrophoresis System according to Pham *et al.* [7]. Staining of the gels was carried out with CBS G250 according to Neuhoff [8]. The derivatization of antibodies with DTPA results in an anodal shift dependent on the chelator content, which could be derived using a calibration curve of conjugates with a known chelator content ranging between 0.5 and 7.0 mol/mol.

Biochemical and immunochemical analysis of DTPA conjugates

DTPA conjugates were assessed for the presence of aggregates by HP-SEC on a Zorbax GF450 column and by SDS-PAGE on 4–26% T, 3% C gradient gels as described by Hoes *et al.* [9]. Immunoreactivity of SC-20 (aCEA)-DTPA conjugates was analysed in a competitive ELISA in CEA-coated microtitration plates using aCEA-HRP as labelled immunoreactant [10]. Immunoreactivity of the 2D11-DTPA conjugates was assessed by cell binding to A431 cells measured by fluorescence activated cell sorter (FACS).

Labelling of the antibody conjugates

For all labelling experiments, suprapure chemicals, ultra-pure water (millipore) and chelex-filtered buffer solutions were used.

^{213}Bi-labelling of immunoconjugates

For the cytotoxicity assays on A431 cells, 250 µg of 2D11-DTPA and of MOPC-DTPA were incubated with 130 µCi of ^{213}Bi in a total volume of 0.5 ml of 0.1 M citrate buffer (pH 5.5). After 15 min at ambient temperature, the reaction mixture was chromatographed on a PD10 column equilibrated in PBS. A sample of the labelled immunoconjugates was chromatographed on a protein G-HiTrap column (Pharmacia) equilibrated in PBS in order to detect the presence of radiocolloids which are not retarded by this affinity column. For the cytotoxicity tests on spheroids, aCEA-DTPA and MOPC-DTPA were labelled according to this procedure with similar results as to radiochemical yield and radiocolloid content.

^{225}Ac-labelling of immunoconjugates

For the cytotoxicity assays on A431 cells, 250 µg of 2D11-DTPA and MOPC-DTPA were incubated with 115 µCi of ^{225}Ac in a total volume of 500 µl of 0.1 M NaCl. After 15 min at ambient temperature, the ^{225}Ac-labelled immunoconjugates were purified and tested as described for the ^{213}Bi-conjugates. For the cytotoxicity test on spheroids, aCEA-DTPA was labelled according to the same procedure with similar results as to radiochemical yield and radiocolloid content.

Calculations

The amount of α-disintegrations per cell was calculated with the aid of the program DIFFEQ, using a K_1 of 0.00095 min^{-1} (binding of antibody/target cells) and a K_2 of 0.0154 min^{-1} (decay of ^{213}Bi). K_1 was estimated by trial and error performing subsequent simulation runs with varying K_1 values until the binding at 1 h was 50% of the maximum.

IN VITRO EXPERIMENTS WITH HUMAN TUMOUR CELL LINES

Cell culturing

The human tumour cell lines A431 and SW 1398, both human carcinoma cell lines, were grown in M505 culture medium supplemented with 10% FCS in cell culture flasks at 37°C/5% CO_2/100% humidity in an incubator. The cells were transferred into fresh culture medium once or twice a week according to the following procedure. After a rinse with PBS, the adherent cells were detached with trypsin in PBS/EDTA for 10 min. After removing the cells with a policeman, they were suspended in culture medium with syringe and needle and the viability was determined with Trypan blue (Gürr, 34078, 1 g l^{-1} in PBS). The cell density was adjusted to 1×10^5 cells ml^{-1} and 10 ml of the suspension was pipetted into new culture flasks (80 cm^2). For assays, the single cell suspension was pipetted into the wells of reaction vials as prescribed.

Preparation of multicell spheroids of SW 1398

Spheroids are cultured in petri dishes coated with a film of agar [5 g l^{-1} of Bacto agar (Difco, 0140-01) dissolved in Milli-Q and sterilized]. SW 1398 multicell spheroids were grown by culturing single cells in agar-coated dishes (10 ml of 1×10^5 cells ml^{-1} $dish^{-1}$) for about 10 days, the culture medium being changed once. The spheroids were then transferred to a 300-ml spinner containing 250 ml of complete culture medium gassed with 5% CO_2 and kept at 37°C. The culture medium was changed once a week by allowing the spheroids to settle and replacing the medium. Spheroids with a diameter less than 200 μm are removed by this method.

Cell-binding assay of labelled conjugates

Single cell suspensions (200 μl) were pipetted into 10-ml polystyrene test tubes at a cell density of 5×10^3, 10^4 and 2×10^4 cells per tube. The cells were pelleted at low speed and the supernatant was removed carefully by suction. The cell pellets were resuspended in 300 μl of PBS containing 0.1 g l^{-1} of ^{213}Bi- and ^{225}Ac-labelled antibodies. Incubation was performed for 45 min at 37°C/5% CO_2/100% humidity. After washing the cells three times with PBS supplemented with 10% FCS, the cells were suspended in PBS/10% and were counted in a gamma counter.

Cytotoxicity assays

A431 cells were pipetted into the wells of a microtitration plate at a density of 5×10^4, 10×10^4 and 20×10^4 cells per well. The plates were incubated overnight at 37°C/5% CO_2/100% humidity for optimal adherence. After careful removal of the culture medium by gentle suction, 25 μl of a dilution series of ^{213}Bi- or ^{225}Ac-labelled antibodies in PBS medium, starting at around 1 μCi per well, were added and incubation was performed for 48 h at 37°C/5% CO_2/100% humidity. Then, 50 μl of a 1 mg ml^{-1} culture medium of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added and the cells were incubated for another 4 h at 37°C/5% CO_2 [10]. The purple formazan crystals formed inside the cells were dissolved in 75 μl DMSO by thorough shaking after removal of the cell culture medium and the absorbance was read at 540 nm in an Anthos ELISA reader. The cytotoxicity of the samples tested was expressed as the percentage of surviving cells related to the number of cells present in the wells to which only PBS was added.

Cytotoxicity assays of ^{225}Ac-labelled aCEA- and MOPC-conjugates on multicell spheroids of SW 1398 cells were performed as follows. Three or four spheroids with diameters ranging between 300 and 700 μm were transferred from the spinner culture into each well of a microtitration plate in 100 μl of cell culture medium. To the spheroids, 25 μl of a dilution series of ^{213}Bi- and ^{225}Ac-labelled immunoconjugates of aCEA and MOPC starting at approximately 1.2–1.3 μCi per well were added and incubation was performed for 2–4 h at 37°C/5% CO_2/100% humidity. Subsequently, the spheroids were washed twice with cell culture medium in order to remove excess, non-bound immunoconjugate and were finally suspended in fresh cell culture medium. Incubation was continued for another 42 h. Viability of the spheroid cells was then determined with MTT as described above.

The cytotoxicity of the samples tested was expressed as a ratio of the percentage survival for the specific (aCEA) and irrelevant (MOPC) conjugate. This ratio ranges between 0 (complete, specific cell-killing) and 1 (complete survival). The formazan absorbance values at 450 nm were corrected for the differences in spheroid size by relating these to the values found for a non-treated control group of spheroids of the same size.

Results and discussion

Synthesis of the ^{213}Bi- and ^{225}Ac-immunoconjugates

An important issue in the synthesis of radioimmunoconjugates is the choice of the chelator, which should allow rapid labelling of the antibodies, while the radionuclide should be stably bound under physiological conditions [11]. For ^{213}Bi, we selected benzyl-DTPA based on the results of Brechbiel and Gansow [12]; this chelator can be introduced into the antibody by reaction of lysine residues with the isothiocyanato-derivative (Fig. 1, compound 1). Compound 1 was synthesized according to the procedure shown in Fig. 1 using *p*-

nitrobenzyl-diethylenetriamine as starting material [13]. By employing the *t*-butyl ester of bromo-acetic acid (instead of the ethyl ester or the free acid) for the introduction of the acetic acid moieties, we were able to synthesize very pure compound 5, since this material can be purified quite easily by chromatography. This material can be stored at −20°C without decomposition and the chelating agent can be generated quite easily by short treatment with the (volatile) trifluoroacetic acid at room temperature. Antibody- and HSA-conjugates synthesized with this chelator usually contained 3–4 moles of benzyl-DTPA per mole of protein.

Derivatization with benzyl-DTPA did not result in detectable oligomerization of the antibodies. The immunoreactivity of the 2D11-conjugates was assessed in a cell-binding assay on A431 cells, analysed by FACS, whereas the immunoreactivity of the SC-20 antibody was tested in an ELISA. In both cases, the immunoreactivity was related to that of the unmodified antibodies. Usually, an immunoreactivity of over 90% was found for both SC-20 and 2D11. The immunoreactivity did not change significantly upon storage at 4°C for at least 3 months, nor was it reduced after binding of the radiolabel in a short period of time (1 h). The conjugates could be labelled with ^{213}Bi in radiochemical yields of 80% and 15 min reaction time under the following conditions:

1. Incubation of the immunoconjugate should be performed at pH 5.5 in the presence of 0.1 M citrate buffer in order to prevent radiocolloid formation. Especially with these heavy metal elements, radiocolloid formation occurs readily and when proper control measurements (i.e. reaction with Cibacron Blue for HSA and protein G for IgGs) are not included, these high molecular complexes can easily be mistaken for radioimmunoconjugates [14].
2. The effective concentration of the conjugated chelator during conjugate labelling should be at least 5 pM, otherwise the yields drop dramatically independent of the amount of ^{213}Bi used. This phenomenon was also observed for isotopes like ^{241}Am and ^{252}Eu (data not shown).
3. The first batch of ^{213}Bi harvested from the ^{225}Ac cows should not be used for labelling experiments, since this material is heavily contaminated with radiation-induced decomposition products of the column matrix used for the ^{213}Bi/^{225}Ac cow.

With the amounts of ^{213}Bi available, radioimmunoconjugates with specific activities up to 3 μCi per μg of IgG could be prepared. About 10% of the radiolabel was not retained on protein G, indicating that only traces of radiocolloids were present. For ^{225}Ac, the situation was more complicated. To date, no suitable ^{225}Ac-chelators for application in immunoconjugates have been described in the literature and, although we began to develop Ac-chelators some years ago, no adequate compound was available at the start of the cytotoxicity experiments.

Though not ideal, we used benzyl-DTPA for ^{225}Ac chelation as well. Under conditions that were slightly different from those for ^{213}Bi, the ^{225}Ac-conjugates with specific activities ranging between 0.4 and 1.0 μCi μg^{-1} were prepared in 80% radiochemical yields in 30 min. As in the case of ^{213}Bi, slightly acidic reaction conditions had to be employed in order to prevent radiocolloid formation; citrate buffer could not be applied because this completely inhibited the formation of the ^{225}Ac-conjugates. Weaker chelating agents such as formate, glycinate or acetate could be used, but optimal radiochemical yields of around 80% were obtained with a 0.1 M NaCl solution. Under optimal conditions, the radiocolloid content was less than 5%. The ^{225}Ac-conjugates were stable in buffer systems but released Ac in the serum-containing cell culture media used for the cytotoxicity experiments (pH 7.2) with a $T_{½}$ of 10–15 min, possibly through transchelation.

^{213}Bi-cytotoxicity experiments

In view of the limited specific activity of the ^{213}Bi-antibodies that could be attained (because of the limited amount of ^{213}Bi available at the time the studies were performed), we required a tumour cell line with a high antigen density for our cytotoxicity studies in order to observe any cell-killing effect at all. We selected a human epidermoid tumour cell line, designated A431, which expresses the EGF-receptor as tumour-associated antigen (at an average of $>10^6$ antigens per cell) and a murine monoclonal antibody, 2D11, directed against a blood group A related carbohydrate structure associated with the EGF-receptor [15]. The antibody MOPC-21 was used as an irrelevant control IgG.

In order to observe cytotoxic effects, the cells had to be incubated continuously with the ^{213}Bi-immunoconjugates. Short incubations were not successful because the binding kinetics of the antibody to the cell surface were too slow in comparison with the incubation time (50% of the antibody was bound after 1 h incubation). Binding of the ^{213}Bi-immunoconjugate was proportional to the number of cells, as shown in Fig. 2. No binding is observed with the irrelevant conjugate. A summary of the cytotoxicity experiments with these ^{213}Bi-labelled antibodies is shown in Fig. 3. These results indicate specific cell-killing with the relevant antibody. No or hardly any non-specific cell-killing is observed with the control antibody, which is not unexpected because the amount of ^{209}Pb (β-emitter) is too low for a cytotoxic effect (dose ~15 cG). Since we measured the amount of ^{213}Bi bound to the cells and the reaction

kinetics of the binding of the antibody to the tumour cells, it was possible to calculate the total amount of disintegrations per cell that brings about 90% cell-killing. These calculations yielded a value of 1000 α-disintegrations per cell, a rather high value in comparison with theoretical calculations where 15 α-disintegrations are needed for 67% single cell-killing and about 1 disintegration per cell in multicell spheroids [16]. However, FACS studies revealed that the A431 cell line used consisted of two populations, of almost equal abundance, with different antigen-expression (i.e. 10^7 *vs* 3×10^5 antigens per cell), as determined in a binding assay with iodinated antibodies (Fig. 4). Correction for heterogeneity in antigen density leads to a value of 30 α-disintegrations per cell for 90% cell-killing, which is in closer agreement with the theoretical values [16]. This means that in practice ^{213}Bi can be considered a 100% α-emitter. Obviously, because of the short half-life of the real α-emitter ^{213}Po (4.2 μs), this radiation dose is directed to the same cells to which the ^{213}Bi-conjugates were originally bound.

Cytotoxicity of ^{225}Ac

The decay chain of ^{225}Ac contains four α-particle emitting daughters (^{221}Fr, ^{217}At, ^{213}Bi, ^{213}Po) and one may question whether these daughter decays contribute to the ^{225}Ac cytotoxicity effects. Even with an ideal Ac-chelator, the recoil energy of the actual Ac decay (~0.1 MeV) is sufficiently high to knock the recoiling ^{221}Fr from the chelator, because this energy surpasses all ionic-binding energies. This was demonstrated in an experiment where ^{225}Ac-ahCG was bound to Sepharose beads coated with hCG and, after removal of excess

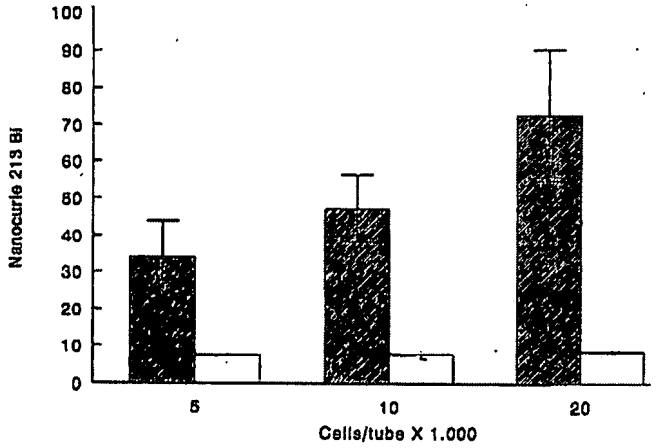

Fig. 2. Binding of ^{213}Bi-labelled immunoconjugates to A431 cells. Binding was studied on 5–20 × 10^3 cells. After incubation for 60 min at 37°C, the cells were washed extensively and ^{213}Bi counts were measured by γ-spectroscopy. Open bars, MOPC; hatched bars, 2D11.

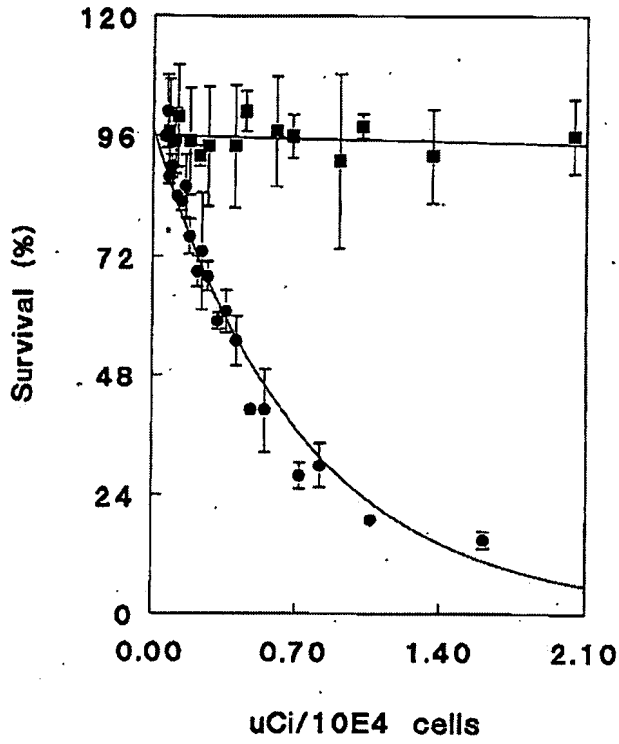

Fig. 3. Cytotoxicity of ^{213}Bi-immunoconjugates on A431 cells. ●, ^{213}Bi-DTPA-2D11 (aEGF); ■, ^{213}Bi-DTPA-MOPC (irrelevant MAb).

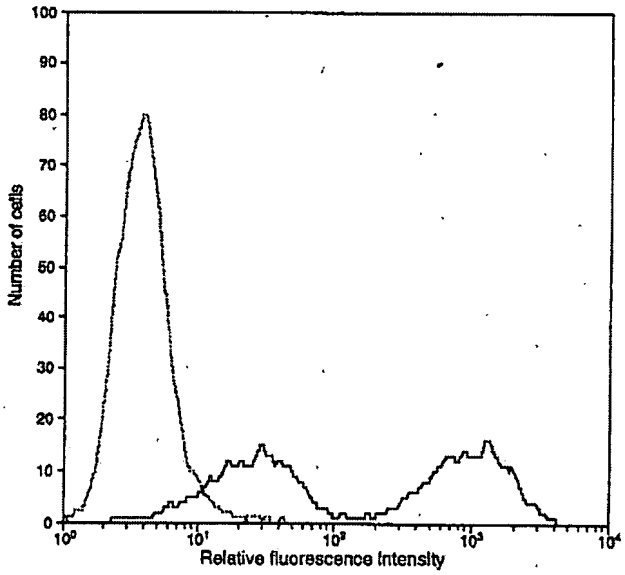

Fig. 4. FACS analysis of binding of 2D11 and MOPC to A431 cells. ——, 2D11; – – – –, MOPC.

unbound antibody, samples were taken from the incubation mixture. These samples contained the almost entire amount of Fr and Bi activities (Ac being virtually completely retained in the solid phase). As a consequence of this recoil, the daughters cannot contribute to the cytotoxic effect of Ac, at least when applied on single cells. This is illustrated by the cytotoxicity of ^{225}Ac-immunoconjugates (Fig. 5). The observed difference with ^{213}Bi (50% less cytotoxic) is in agreement with the calculated value (45%) using one α-decay, the same binding kinetics as for the ^{213}Bi-conjugates, and a $T_{1/2}$ of 15 min for release of ^{225}Ac from the conjugates in the incubation medium. Part of the ^{225}Ac released from the immunoconjugates is bound or taken up by the cells, as is evident from the radioactivity present in the cell pellet incubated with the irrelevant immunoconjugate. This accounts for the cytotoxicity on the cells treated with ^{225}Ac-MOPC conjugate, which significantly differs from the values found for cells incubated with buffer (Fig. 5).

We also studied the cytotoxic effects of the immunoconjugates in multicell spheroids, a more realistic model for the *in-vivo* situation. Spheroids of SW 1398 cells were incubated with the labelled immunoconjugates (SC-20, a murine IgG) directed against the tumour antigen CEA (in which time the antibodies had migrated to the centre of the spheroids, measured by histochemical analysis) for 4 h at most. After washing, the spheroids were incubated for 48 h in cell culture medium, after which period the percentage survival was measured with MTT. The results are presented in Fig. 6. For ^{213}Bi, no specific cell-killing was observed, which is an indication for the limited applicability of this radionuclide in the treatment of solid tumours [17]. For ^{225}Ac, on the other hand, specific cell-killing was measured under identical experimental conditions (same amount of radioactivity/spheroid), suggesting possible application of ^{225}Ac-immunoconjugates in the treatment of solid tumours (metastases). In view of the limited stability of ^{225}Ac chelation, the cytotoxicity observed cannot be explained by the longer half-life of ^{225}Ac (and as a consequence the number of α-decays per μCi), but suggests an additional effect of the Ac-daughters in areas distal from the immunoconjugate binding site (field effect). Because of their (relatively short) half-lives, the Ac-daughters will diffuse away from the original target cells but containment within the spheroids is likely. Further experiments using a better Ac-chelator will be performed to prove this point.

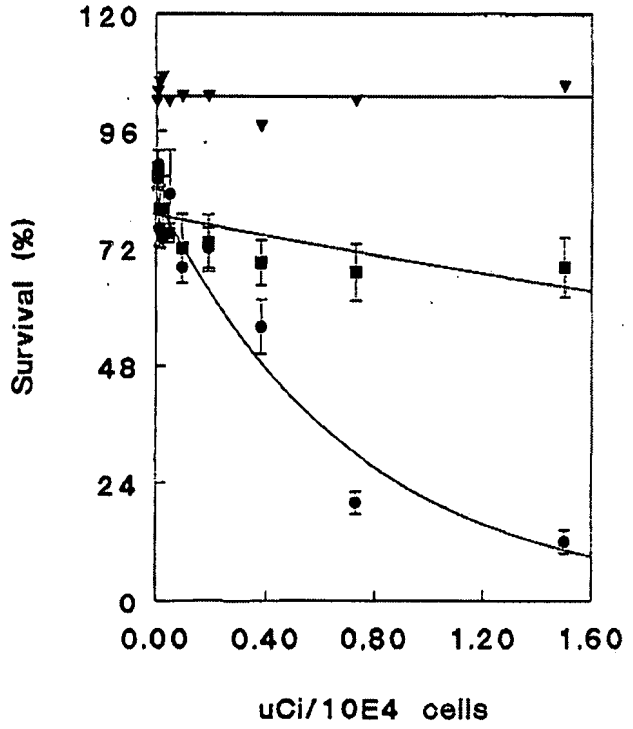

Fig. 5. Cytotoxicity of ^{225}Ac-immunoconjugates on A431 cells. ▼, no labelled antibodies; ■, ^{225}Ac-DTPA-MOPC; ●, ^{225}Ac-DTPA-2D11.

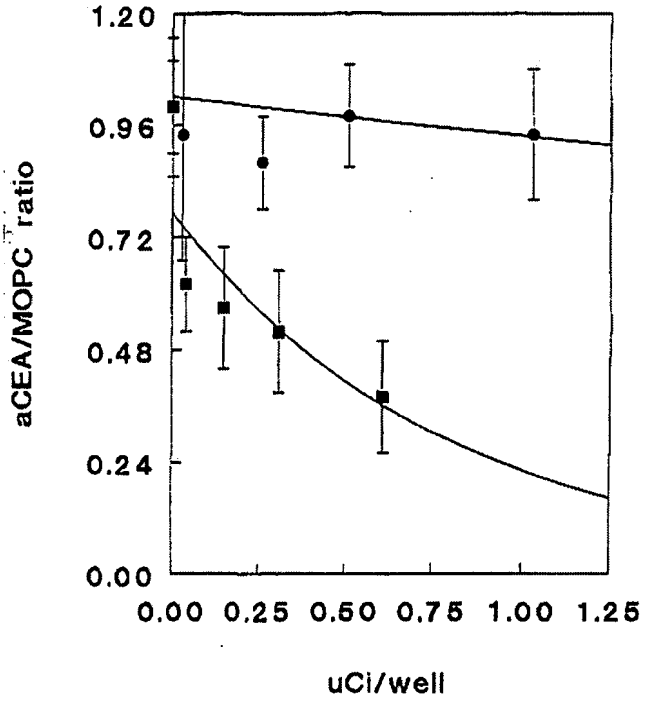

Fig. 6. Cytotoxicity of ^{213}Bi- and ^{225}Ac-immunoconjugates on SW 1398 spheroids. The cytotoxicity is expressed as the ratio of survival on treatment with a relevant (aCEA) and an irrelevant (MOPC) antibody. ●, ^{213}Bi; ■, ^{225}Ac.

Conclusions

The experiments described above justify further preclinical studies with ^{225}Ac and ^{213}Bi in the immunotherapy of cancer. ^{213}Bi can be regarded as an alternative to ^{212}Bi in the treatment of single-cell malignancies, with the advantage of safer and easier production [1]. One drawback could be the low dose of the β-emitter ^{209}Pb, formed as a result of the decay, but intravenous administration of a scavenger like EDTA might help overcome this problem [18]. The results presented here on the cytotoxicity of ^{213}Bi-immunoconjugates are in agreement with the data obtained with ^{211}At-labelled monoclonal antibodies by Larsen *et al.* [19]. Under comparable experimental conditions as regards specific activity of conjugates and activity levels in culture medium, they found efficient cell-killing on various osteosarcoma cell lines and microcolonies, the cytotoxic efficiency being dependent mainly on the antigen density of the cell line employed.

^{225}Ac is a candidate for the treatment of solid tumours, since problems of α-treatment in this type of tumour, due to the heterogeneity of antigen expression, may be overcome by the field effect of the α-emitting daughters of ^{225}Ac. For a therapeutic effect in humans, an injected dose of 5 mg of antibody would suffice, assuming an antigen density of 5×10^4 antigens per cell, a specific activity of 10 mCi mg^{-1} and a targeting efficiency of 0.01% g^{-1}. Obviously, we require optimal pharmacokinetics, in particular for ^{213}Bi-conjugates, in view of the short half-life of the radionuclide. However, the design of a suitable chelator for ^{225}Ac is essential for such an application.

Acknowledgements

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Radioimmunotherapy of Peritoneal Human Colon Cancer Xenografts with Site-specifically Modified ^{212}Bi-labeled Antibody[1]

R. Bruce Simonson, Michiel E. Ultee,[2] Jo A. Hauler, and Vernon L. Alvarez

CYTOGEN Corporation, Princeton, New Jersey 08540

Abstract

^{212}Bi is a radioisotope that emits highly cytotoxic α-particles. α-particles have a high linear energy transfer over a short path length. These properties and the 1-h half-life make this isotope suitable for radioimmunotherapy of peritoneal tumors. Therefore, we wanted to test whether monoclonal antibodies labeled with ^{212}Bi would be effective in treating such tumors. We conjugated the antibody B72.3, which is reactive with many human adenocarcinomas, to the chelator linker glycyltyrosyllysyl-*N*-ϵ-diethylenetriaminepentaacetic acid, by reductive amination to the carbohydrate residues of the antibody (J. Rodwell, *et al.* Proc. Natl. Acad. Sci. USA, 83: 2632–2636, 1986). Athymic nude mice were injected i.p. with LS174T cells, a human colon cancer cell line. Seven to 13 days later the mice were treated with the ^{212}Bi-labeled antibody. We treated the mice using single doses of 180–450 μCi or multiple doses of 80–180 μCi on consecutive days. Dissections were performed 9–16 days after the end of treatment. Both the single and multiple doses resulted in a decrease in tumor burden when compared to tumor from mice receiving unlabeled antibody. Mice in the optimum group showed tumor reductions of greater than 90%. Treatment with a ^{212}Bi-labeled irrelevant antibody was significantly less effective than that with labeled B72.3 antibody. Survival studies showed that mice receiving the labeled antibody had a prolonged survival when compared to control mice.

Introduction

Targeting tumors with radioisotopes attached to specific monoclonal antibodies is an active area of cancer research (1, 2). The most common radioisotopes used for radioimmunotherapy are ^{131}I and ^{90}Y, both of which emit β-particles with energetics and half-lives favorable for therapy (3).

Isotopes emitting α-particles offer several advantages over β-emitters (2–4): (*a*) their high linear energy transfer (100 KeV/μm) render them very cytotoxic; (*b*) since the path length of α-particles is only 40–80 μm, the nonspecific irradiation of normal tissue around the targeted tumor is greatly reduced; (*c*) the DNA damage caused by α-particles is not easily repaired by the cell; (*d*) the cytotoxicity of α-particles is not affected by oxygen, and therefore they are capable of killing cells under hypoxic conditions.

The α-emitters with the most promise for radioimmunotherapy are ^{211}At and ^{212}Bi (2). Astatine is a halogen with an even weaker bond to carbon than iodine and would be unstable with respect to dehalogenation *in vivo* if linked by conventional chemistry. Furthermore, it must be prepared in a cyclotron and is not commercially available. Nevertheless, it has been used successfully by some investigators (5, 6). ^{212}Bi can be coupled to antibodies using familiar metal chelates such as DTPA[3] and can be easily eluted from a generator available from Argonne National Laboratory (7).

While the 1-h half-life of ^{212}Bi limits its therapeutic applications, it might be especially appropriate when delivered i.p. for treatment of localized cancer. Recent clinical studies have indicated the this route of injection may be especially suitable for peritoneal implant metastases (8) and ascitic cancer (9).

In this study we site-specifically labeled a monoclonal antibody with ^{212}Bi and tested its therapeutic potential in an i.p. xenograft model (10). The antibody chosen was B72.3, a murine IgG1 that recognizes the mucin antigen TAG-72, found on many human adenocarcinomas (11).

The cell line used was LS174T, a human colon carcinoma (12). These cells were grown i.p. in athymic nude mice and the mice were treated with the ^{212}Bi-labeled antibody.

Materials and Methods

i.p. Xenograft Model. We followed the procedure of Hyams *et al.* (10), except that we found that pretreatment of the mice with anti-mouse T-lymphocyte antisera was without effect and therefore unnecessary. Athymic nude mice (*nu/nu* Swiss; Taconic Farms, Germantown, NY) were given i.p. injections of 1.0×10^6 LS174T cells (American Type Culture Collection, Rockville, MD) in 1 ml of 10 mM phosphate-150 mM NaCl, pH 7.4. Autopsies were performed on random mice 8 days after tumor cell inoculation to verify the presence of tumor nodules.

Preparation of Antibody-Linker Chelator. The B72.3 (Celltech, United Kingdom) and BL3 (13) (Damon Biotech, Needham Heights, MA) antibodies were conjugated site-specifically via their carbohydrate to the linker-chelator GYK-DTPA, as described by Alvarez *et al.* (14).

Labeling of Antibody-GYK-DTPA with ^{212}Bi. We eluted the ^{212}Bi from a ^{224}Ra generator using 0.15 M hydroiodic acid, following the procedure of Atcher *et al.* (7). The decay kinetics and energy profile on a multichannel analyzer (EGG Ortec, Oak Ridge, TN) identified the eluted material as ^{212}Bi. After neutralization with sodium acetate, the ^{212}Bi was combined with 100–200 μg of antibody-GYK-DTPA conjugate. The sample was incubated at 37°C for 15 min, which we found to be the optimal time for maximum labeling. Free ^{212}Bi was removed by chromatography of the antibody conjugate on a TSK-G3000 SW column (Phenomenex, Rancho Palos Verdes, CA).

The amount of ^{212}Bi attached to the antibody was determined using the multichannel analyzer.

Radioimmunotherapy of i.p. Xenografts. Seven to 13 days after tumor cell inoculation, the mice were given i.p. injections of B72.3-GYK-DTPA-^{212}Bi. As controls, we used BL3-GYK-DTPA-^{212}Bi and 10 mM phosphate-150 mM NaCl, pH 7.4, and antibody alone. Both single and multiple dosings were performed, with the multiple doses given over consecutive days.

The ^{212}Bi dose ranged from 90 to 450 mCi, with the injected volume from 0.4 to 0.6 ml. The specific activity of the ^{212}Bi-labeled antibody was 5–10 μCi/μg.

Mice were weighed daily on weekdays during the studies. With the exception of those used for survival studies, the mice were sacrificed 22–25 days after tumor cell inoculation. Tumor nodules were carefully dissected and weighed, and any ascites formed was volumetrically measured. For survival studies the mice were kept in a sterile environment and checked daily for survival.

Results

i.p. Model. In this model the LS174T cells grew as both solid tumor and ascites. We observed ascites formation beginning about 20 days after injection of the tumor cells. At time of dissection, the LS174T tumor had spread over the internal organs and along the lining of the peritoneum. The amount of

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: DTPA, diethylenetriaminepentaacetic acid; GYK-DTPA, glycyltyrosyllysyl-*N*-ϵ-diethylenetriaminepentaacetic acid.

ascites ranged from trace amounts to as high as 4.0 ml.

^{212}Bi Labeling of the Antibody-GYK-DTPA. Using high performance liquid chromatography to remove the unbound ^{212}Bi, we determined that greater than 75% of the ^{212}Bi added to the reaction was bound by the antibody conjugates. The specific activity of the ^{212}Bi-labeled conjugates ranged from 5 to 10 μCi/μg. Incubation of the labeled conjugate in fresh serum for as long as 4 h (4 half-lives) did not dissociate the label (data not shown).

i.p. Tumor Therapy. We first looked at the effect of different doses of ^{212}Bi-labeled B72.3 conjugate on the LS174T tumor. Mice were treated with the ^{212}Bi antibody 13 days after the LS174T tumor cells were injected. One group of mice received a single 190-μCi dose, while a second group received a higher 450-μCi dose. A third group received 190-μCi doses on 3 consecutive days, and control mice received unlabeled B72.3-GYK-DTPA.

The results of the dissection done 12 days following antibody treatment are shown in Fig. 1. Mice receiving the single 190-μCi dose showed a slight decrease in weight of tumor dissected when compared to the control. Those receiving the single 450-μCi dose or the multiple 190-μCi dose showed a 56% decrease in tumor mass when compared to the control.

This first study indicated that the ^{211}Bi-labeled antibody reduced the tumor growth in the mice. However, the tumors were well advanced (13 days) at the start of the study. Therefore, in later studies we switched to 7–8-day-old tumors. Although the single large dose and multiple smaller doses gave similar tumor reduction, we continued with multiple doses for reasons of convenience in eluting the ^{212}Bi generator.

Fig. 2 shows the results of two different multiple dosing levels. One group of mice received four 90-μCi doses on consecutive days while a second group received four 180-μCi doses on consecutive days. The ^{212}Bi-labeled antibody was injected starting 8 days after the injection of the tumor cells. Dissections were performed 14 days after the end of the treatment. One of the four mice that received the consecutive 90-μCi doses did not have any observable tumor. The remaining mice in the group averaged 85% reduction in tumor burden when compared to the mice that received the unlabeled antibody. The high dose was even more effective. Three of four mice that received it had no observable tumor, while the fourth mouse had only 20 mg of tumor.

Toxicity of the ^{212}Bi. Almost all of the mice that received the ^{212}Bi-labeled antibody exhibited some radiotoxicity. This was characterized by visible dehydration and loss of weight. Fig. 3 shows the percentage weight change of the mice that received

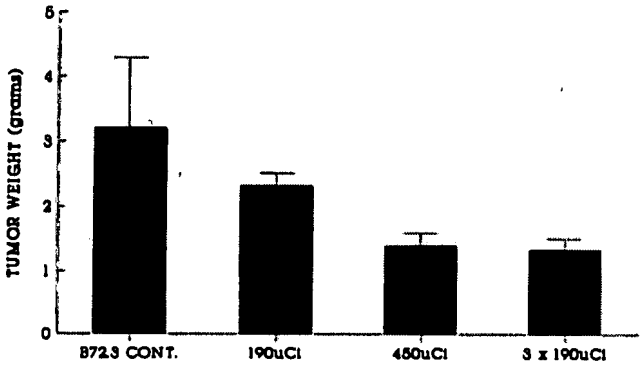

Fig. 1. Reduction of i.p. LS174T tumor using single and multiple doses of ^{212}Bi-labeled B72.3-GYK-DTPA. *Columns*, average tumor mass from four mice per group; *bars*, SD.

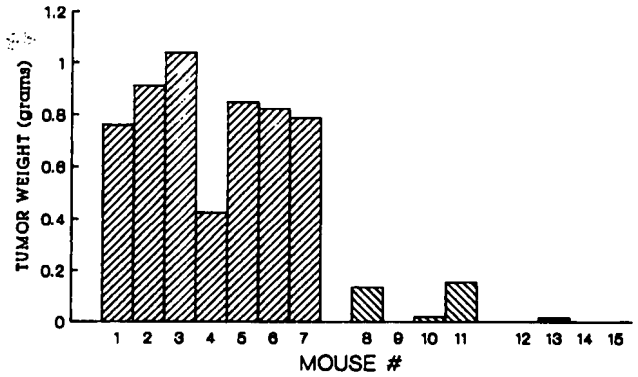

Fig. 2. Reduction of i.p. LS174T tumor using multiple doses of ^{212}Bi-labeled B72.3-GYK-DTPA. Multiple doses of 90 μCi (▨, mice 8–11) and 180 μCi (■, mice 12–15) were compared to mice receiving unlabeled control (▨, mice 1–7). *Ordinate*, tumor weights of individual mice in each group.

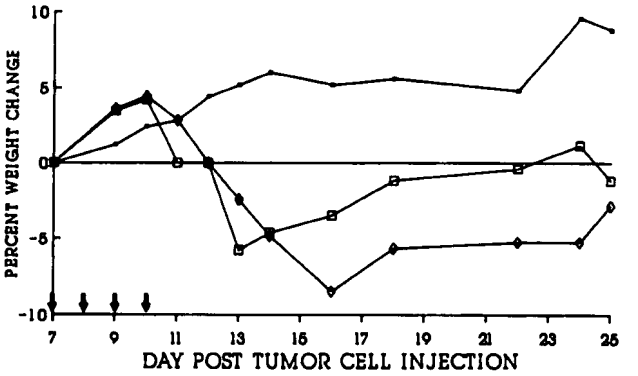

Fig. 3. Radiotoxicity of ^{212}Bi-labeled B72.3-GYK-DTPA, as measured by percentage weight change of the treated mice. Multiple doses of 90 μCi (□) and 180 μCi (◇) were compared with the unlabeled B72.3 (■). *Arrows*, days of treatment.

multiple doses of 90 μCi, 180 μCi, and unlabeled antibody. The mice receiving the 90-μCi dose lost about 5% of their body weight 3 days after the end of treatment. Starting at day 14 the mice began to gain their weight back and the dehydration lessened. The mice receiving the multiple 180-μCi doses showed more toxic effects of the ^{212}Bi, losing about 9% of their body weight 6 days after the end of treatment. As with the lower dose, after this point the treated mice began to gain back the lost weight and the dehydration lessened.

Determination of Antibody-specific Killing. To determine if the decrease in tumor burden was antibody specific, ^{212}Bi-labeled B72.3 antibody was compared with a commercially available, isotype-matched, irrelevant antibody, BL3 (13). Mice received three 120-μCi doses of either the B72.3- or BL3-labeled antibody on consecutive days. The results of the dissection are presented in Fig. 4. There is a dramatic difference in the average tumor burden of the mice that received the ^{212}Bi-labeled B72.3 compared to that of the mice that received the ^{212}Bi-labeled BL3 conjugate.

Survival Study. Mice were given injections 7 days after the injection of LS174T cells with the ^{212}Bi-labeled B72.3. One group of mice received 100 μCi injections on 3 consecutive days. A second group received 150-μCi doses also on 3 consecutive days. A third group received 3 injections of unlabeled B72.3 conjugate. Fig. 5 shows the survival curve for these mice. All the control mice died by day 68, while some of the mice receiving the ^{212}Bi-labeled antibody showed prolonged survival.

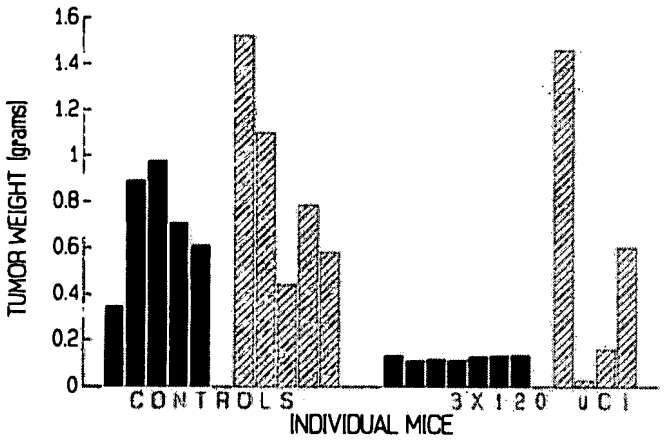

Fig. 4. Effect of ^{212}Bi-labeled B72.3 (■) compared to the irrelevant antibody BL3 (▩). Mice received injections of the respective antibody without (controls) or with (3 × 120 μCi) ^{212}Bi on consecutive days.

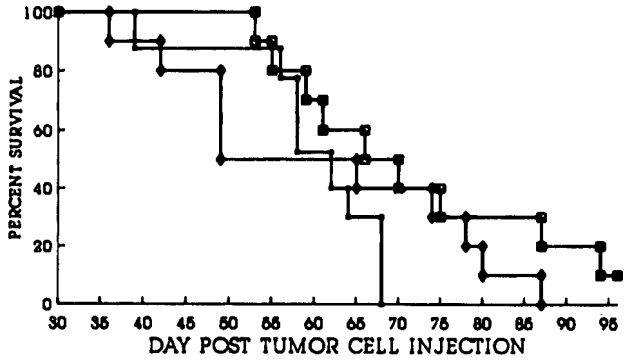

Fig. 5. ^{212}Bi-labeled B72.3 therapy of i.p. LS174T tumor. Survival of mice (10/group) treated with unlabeled antibody (■), 3 × 100 μCi doses (◇) and 3 × 150 μCi doses (□) on consecutive days.

Discussion

^{212}Bi is an α emitting radioisotope that has properties that make it suitable for radioimmunotherapy. The short half-life of ^{212}Bi limits its use *in vivo*. One possible use would be in an i.p. injection in therapy against peritoneal disease, such as ovarian carcinoma (8, 9).

We treated mice bearing LS174T tumor with ^{212}Bi-labeled B72.3 antibody. The B72.3 was modified through the carbohydrate with the linker-chelator GYK-DTPA. Both single and multiple dosing of the ^{212}Bi labeled antibody resulted in substantial decrease of the LS174T tumor burden when compared to mice that received injections of unlabeled antibody.

In mice that received the multiple 180-μCi doses, three of the four mice did not have any observable tumor upon dissection. Multiple dosing at radiation levels of one-half this amount still resulted in significant decreases in the amount of tumor.

The mice receiving the ^{212}Bi-labeled antibody did show some radiotoxicity, in terms of dehydration and weight loss. In most cases the weight loss was less than 10% and was only temporary. Within 2 weeks after the end of treatment, these signs of radiotoxicity were gone.

The reduction of the LS174T tumor is mostly antibody mediated. This was shown when the effect of the relevant antibody B72.3 was compared to the irrelevant antibody BL3. The labeled BL3 antibody did have some effect. This nonspecific kill could be expected due to the model, since the labeled antibody would bathe the peritoneum.

Treatment of the tumor-bearing mice with the ^{212}Bi-labeled antibody resulted in a prolonged survival for some of them when compared to the mice receiving the unlabeled antibody. However, we did not obtain any cures for the tumor-bearing mice. We believe several factors reduced the efficacy of our ^{212}Bi antibody. First, the B72.3 antibody recognizes the mucin antigen TAG-72 (15), which is secreted rather than bound to the cell surface. Since the α-particles travel such a short distance, the effectiveness of ^{212}Bi treatment would have been better had we used a monoclonal antibody that recognized an antigen bound to the cell surface. Secondly, in our experiments treatment with the ^{212}Bi-labeled antibody was not begun until at least 7 days after the injection of the LS174T cells. By this time the tumor was well established. Finally, the mice developed ascites only after the development of solid LS174T tumor. The ^{212}Bi-labeled antibody would probably be more effective in a model in which ascites developed earlier.

In a recent work Macklis *et al.* (16) were able to cure mice of an i.p. tumor using antibody labeled with ^{212}Bi. We see three key differences between our studies: (*a*) they treated the tumor-bearing mice only 1 day after the tumor cell inoculation, rather than 7–13 days after; (*b*) the antibody they used was anti-Thy-1.2, which recognizes a membrane antigen rather than a secreted one; (*c*) they treated immunocompetent normal mice bearing murine lymphomas, rather than athymic nude mice bearing human cancer xenografts.

One problem with the chelator-linker GYK-DTPA is the *in vivo* stability of the chelated ^{212}Bi. Within a few h after i.p. injection, about 20% of the injected ^{212}Bi is found in the kidney (data not shown). A stronger chelator for the ^{212}Bi would be needed to prevent this. Although isothiocyanate derivatives of DTPA (17) are reported to be more stable chelators for bismuth, their antibody conjugates still show *in vivo* instability.[4]

One alternative to chelating ^{212}Bi directly to monoclonal antibodies would be to chelate its decay parent, ^{212}Pb. Its 10.6-h half-life and relationship to ^{212}Bi make it a convenient route for delivering ^{212}Bi over a longer time than the 1-h half-life of ^{212}Bi allows (2, 4). This would be needed for efficient targeting of solid tumors by ^{212}Bi. However, to date no suitable chelator has been reported for attaching lead to antibodies.

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